Eradication of *Citrus tristeza virus* from cultivar Zorica Rana (*Citrus unshiu* Marc.)

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Introduction

Varieties of Satsuma mandarin are commercially the most important citrus crop in Croatia due to their relative frost tolerance. *Citrus tristeza virus* (CTV) is the causal agent of tristeza, the most devastating viral disease of citrus. CTV has been detected in most of the Satsuma mandarin (*Citrus unshiu* Marc.) varieties including different and even the newest clones of cv. Zorica Rana. The detailed procedure for CTV elimination from different citrus species has already been described and developed (Navarro *et al.*, 1975; Starrantino and Caruso, 1988; Singh, 2001) but not in case of Satsuma mandarin cv. Zorica Rana. Raising virus-free plants is an important aspect of citrus cultivation in Croatia.

Materials and methods

Two-year old Satsuma mandarin plants cv. Zorica Rana from collection orchard that were previously shown to be CTV positive (ELISA-test and IC/RT-PCR) were selected for virus elimination. After three months of thermotherapy plants of three clones were used as a source of explants for shoot-tip grafting (micrografting) on Troyer citrange rootstock germinated *in vitro* following the method essentially described by Navarro *et al.* (1975). The original procedure was modified in performing grafting procedure ("L" incision) and nutrition media composition. Clones: 3/21-01, 5/19-01, 9/12-01 and 5/23-01 were also subjected to CTV biological detection on two-year old seedlings of Mexican lime (*Citrus aurantifolia* L.), CRC Grapefruit (*Citrus paradisi* Macf.), Sour Orange (*Citrus aurantium* L.) and Madame Vinous Sweet Orange [*Citrus sinensis* (L.) Osbeck]. Micrografted plants were tested for the virus by DAS-ELISA (*Double Antibody Sandwich Enzyme Linked Imunosorbent Assay*, Clark and Adams, 1977) using commercial antisera according to the manufacturers protocols (Agritest, Italy) and IC/RT-PCR (*Immunocapture Reverse Transcription Polymerase Chain Reaction*, Zemzami *et al*., 2002) using primers for the CTV coat protein gene (Nolasco *et al.*, 1993). The same diagnostic methods were utilized for testing indicator plants.

Results and discussion

Observation of morphological changes and appearance of viral disease symptoms on citrus indicator hosts in comparison with positive and negative control plants had confirmed the presence of CTV in all tested clones of cv. Zorica Rana. Leaf cupping, chlorosis and vein enation were observed in Mexican lime seedlings only two months after grafting. Symptoms of stem-pitting (SP) started to appear on Mexican lime after seven months and, in the case of clone 5/23-01, in CRC Grapefruit seedlings, too. Lime reaction produced by CTV isolate from the mandarin clone 5/23-01 was much stronger in comparison with the other tested clones (growth retardation). No symptoms that could be attributed to CTV infection were observed in Sweet and Sour Orange indicators which suggests the presence of mild to moderate SP-CTV strains in 'Zorica Rana' clone 5/23-01. Biotest results imply biological diversity among detected CTV isolates. The presence of virus tristeza determined by biological indexing in tested clones has been positively correlated with CTV infection previously diagnosed by DAS-ELISA and IC/RT-PCR (Škorić *et al*., 2005).

CTV-free plants of three ‘Zorica Rana’ clones were obtained by thermotherapy in combination with shoot-tip grafting *in vitro* from infected plants. Following this procedure, 20% of plants were tristeza-negative. Many factors influence the recovery of micrografted plants. The highest rate of successful grafts was obtained with 2-week old Troyer citrange seedlings (Navarro, 1992), what is in agreement with our results. Degree of tissue differentiation of the rootstock, which is affected by light and age (Navarro, 1992) is also of great importance. The best results were obtained by grafting at the top of the decapitated epicotyl, placing the shoot-tip in contact with the vascular ring (Navarro *et al.* 1975). In our experiment, we placed shoot-tip in the “L” incision on the rootstock, which was also more practical to perform than inverse “T” incision commonly used in most laboratories (Singh, 2001). Additional pre-treatment with growth regulators before shoot-tip isolation could increase the incidence of successful grafts (Starrantino and Caruso, 1988).

After shoot-tip grafting, successfully cultured plants were established *in vivo*, this resulted in ninety percent survival and excellent subsequent growth.

Further sanitation of infected important cultivars of Satsuma mandarin, as well as other citrus species, is a prerequisite for obtaining high quality foundation plant material. Micrografting *in vitro* may also have an important role in basic research for graft compatibility, physiology of the graft union and plant aging.

Conclusions

The appearance of different symptoms in biological indicators suggests biological diversity among detected CTV isolates in investigated ‘Zorica Rana’ mandarin clones. *In vitro* grafting of small (0.3 mm) shoot tips on 2-week old Troyer Citrange rootstock seedlings enables successful grafts that were transplanted to soil with 90% survival rate. The efficiency of virus tristeza eradication from cv. Zorica Rana Satsuma mandarin was tested by using laboratory diagnostic methods DAS-ELISA and IC/RT-PCR. CTV elimination was confirmed in 20% of tested plants.

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